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Geomyces destructans sp. nov. associated with bat white-nose syndrome

A. Gargas[1], M.T. Trest[2], M. Christensen[3]
T.J. Volk[4] & D.S. Blehert[5*]

[1]*andreagargas@symbiology.com*
Symbiology LLC
Middleton, WI 53562 USA

[2]*mttrest@wisc.edu*
Department of Botany, University of Wisconsin — Madison
Birge Hall, 430 Lincoln Drive, Madison, WI 53706 USA

[3]*martchris@att.net*
1713 Frisch Road, Madison, WI 53711 USA

[4]*volk.thom@uwlax.edu*
Department of Biology, University of Wisconsin — La Crosse
3024 Crowley Hall, La Crosse, WI 54601 USA

[5]*dblehert@usgs.gov*
U.S. Geological Survey — National Wildlife Health Center
6006 Schroeder Road, Madison, WI 53711 USA

Abstract — We describe and illustrate the new species *Geomyces destructans*. Bats infected with this fungus present with powdery conidia and hyphae on their muzzles, wing membranes, and/or pinnae, leading to description of the accompanying disease as white-nose syndrome, a cause of widespread mortality among hibernating bats in the northeastern US. Based on rRNA gene sequence (ITS and SSU) characters the fungus is placed in the genus *Geomyces*, yet its distinctive asymmetrically curved conidia are unlike those of any described *Geomyces* species.

Key words — *Ascomycota*, *Helotiales*, *Pseudogymnoascus*, psychrophilic, systematics

Introduction

Bat white-nose syndrome (WNS) was first documented in a photograph taken at Howes Cave, 52 km west of Albany, NY USA during winter, 2006 (Blehert et al. 2009). As of March 2009, WNS has been confirmed by gross and histologic examination of bats at caves and mines in Massachusetts, New Jersey, Vermont, West Virginia, New Hampshire, Connecticut, Virginia, and Pennsylvania. The

*Corresponding Author.

syndrome is characterized by the presence of profuse yet delicate hyphae and conidia on bat muzzles, wing membranes, and/or pinnae, although these surface signs are readily removed. Histological examination of infected bats shows that fungal hyphae pervade the bat tissue filling hair follicles and sebaceous glands, yet the fungus does not typically lead to inflammation or immune response in the tissue of hibernating bats (Meteyer et al. 2009).

Through April 2009, the WNS fungus has been isolated from four species of bats including little brown (*Myotis lucifugus* Le Conte), northern long-eared (*Myotis septentrionalis* Trovessart), big brown (*Eptesicus fuscus* Beauvois), and tricolored bats (*Perimyotis subflavus* Menu). Initial analysis of small subunit (SSU) and internal transcribed spacer (ITS) rRNA gene sequences placed this fungus into the genus *Geomyces*. The fungus produces arthroconidia on verticillately branched conidiophores and on prostrate hyphae, typical of genus *Geomyces,* but the asymmetrically curved conidia are unlike any described species.

Materials and methods

Isolation & culture

We isolated the fungus from eight infected bats, representing two species, collected in four US states between January and April of 2008 (Table 1). Wing tissue was placed directly onto Sabouraud dextrose agar (Sab dex; BD Diagnostics, Franklin Lakes, NJ) and incubated at 3°C for initial isolation of the fungus. Growth characteristics were determined on cornmeal agar (CMA; Difco, Detroit, MI), with temperature tolerance determined by incubation at 7°C, 14°C, or 24°C.

Table 1. *Geomyces destructans* isolates examined.

NWHC case #	Bat (*Myotis*) species	Coll. date	Coll. location	GenBank accession # ITS region	GenBank accession # SSU region
20631-21 T*	*M. lucifugus*	2 Feb 2008	Williams Hotel, NY	EU884921	FJ231098
20631-8 PT	*M. lucifugus*	29 Jan 2008	Hailes Cave, NY	EU884920	FJ231097
20674-9 PT	*M. septentrionalis*	18 Mar 2008	Aeolus Cave, VT	FJ170115	FJ231093
20674-10 PT	*M. lucifugus*	18 Mar 2008	Aeolus Cave, VT	EU884922	FJ231094
20682-1 PT	*M. septentrionalis*	21 Mar 2008	Berkshire Co., MA	EU854570	FJ231094
20682-10 PT	*M. septentrionalis*	21 Mar 2008	Berkshire Co., MA	EU854569	FJ231095
20693-1 PT	*M. lucifugus*	26 Mar 2008	Chester Mine, MA	EU884923	FJ231096
22004-1 PT	*M. lucifugus*	1 Apr 2008	Litchfield Co., CT	EU884924	FJ231093

*BPI878935, holotype (T); PT = paratype.

NWHC = US Geological Survey — National Wildlife Health Center

Sequencing & analysis

DNA was extracted from the fungal isolates following the manufacturer's instructions for microLYSIS-PLUS reagent (The Gel Company, San Francisco, CA). We used primers

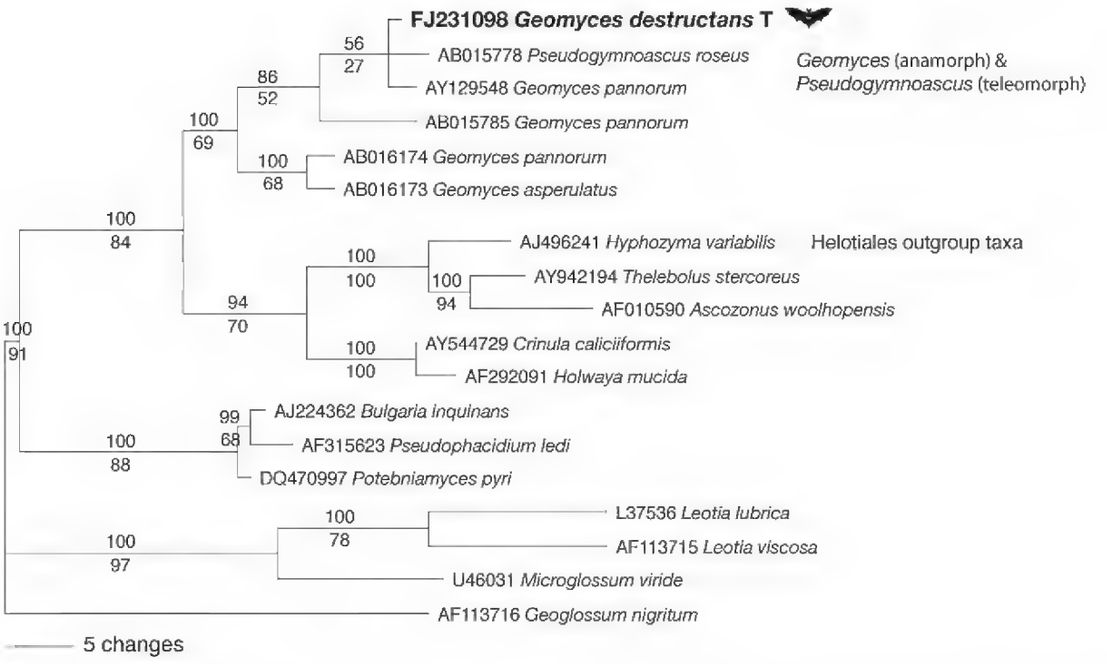

FIG. 1. One of 5 equally parsimonious trees for the SSU alignment (Length = 194, CI = 0.825, RI = 0.807). GenBank accession numbers precede taxon names, the sequence from *Geomyces destructans* is indicated in bold with a bat image, and the type isolate is indicated with a bold capital T. Branch length is relative to the number of substitutions per site. Posterior probability values are shown above each supported node, and bootstrap percentages are shown below supported nodes. Modified from Blehert et al. (2009) online supplement.

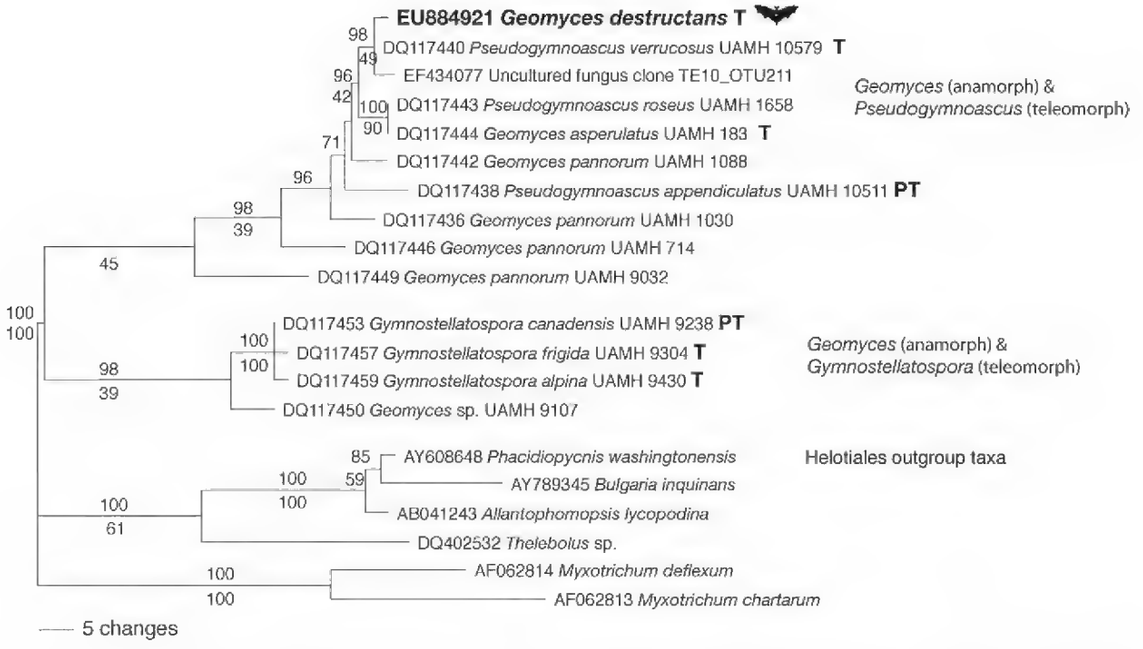
EU884921 Geomyces destructans T
DQ117440 Pseudogymnoascus verrucosus UAMH 10579 T
EF434077 Uncultured fungus clone TE10_OTU211
DQ117443 Pseudogymnoascus roseus UAMH 1658
DQ117444 Geomyces asperulatus UAMH 183 T
DQ117442 Geomyces pannorum UAMH 1088
DQ117438 Pseudogymnoascus appendiculatus UAMH 10511 PT
DQ117436 Geomyces pannorum UAMH 1030
DQ117446 Geomyces pannorum UAMH 714
DQ117449 Geomyces pannorum UAMH 9032
Geomyces (anamorph) & Pseudogymnoascus (teleomorph)
DQ117453 Gymnostellatospora canadensis UAMH 9238 PT
DQ117457 Gymnostellatospora frigida UAMH 9304 T
DQ117459 Gymnostellatospora alpina UAMH 9430 T
DQ117450 Geomyces sp. UAMH 9107
Geomyces (anamorph) & Gymnostellatospora (teleomorph)
AY608648 Phacidiopycnis washingtonensis
AY789345 Bulgaria inquinans
AB041243 Allantophomopsis lycopodina
DQ402532 Thelebolus sp.
Helotiales outgroup taxa
AF062814 Myxotrichum deflexum
AF062813 Myxotrichum chartarum
5 changes

FIG. 2. One of 13 equally parsimonious trees for the ITS alignment (Length = 286, CI = 0.734, RI = 0.805). GenBank accession numbers precede taxon names, and the sequence from *Geomyces destructans* is indicated in bold with a bat image. Type isolates are indicated by bold capital Ts and paratype isolates by bold capital PTs. Branch length is relative to the number of substitutions per site. Posterior probability values are shown above each supported node, and bootstrap percentages are shown below supported nodes. Modified from Blehert et al. (2009) online supplement.

ITS4 and ITS5 (White et al. 1990) and ExTaq proof-reading DNA polymerase (Takara Mirus Bio, Madison, WI) to PCR amplify the rRNA gene ITS region (ITS1, 5.8S, and ITS2). PCR cycling parameters included an initial 2 min denaturation at 98°C, then 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. The rRNA gene SSU was amplified as above, but using primers nu-SSU-0021-5' (Gargas & DePriest 1996) and nu-SSU-1750-3' (Gargas & Taylor 1992) and an increased extension time of 2 min. Sequencing primers were the same as PCR primers with the addition of nu-SSU-0402-5' (Gargas & Taylor 1992), nu-SSU-1150-5' (White et al. 1990), nu-SSU-0497-3' (Gargas & Taylor 1992), and nu-SSU-1184-3' (Gargas et al. 1995) for the SSU.

PCR products were sequenced by the University of Wisconsin — Madison Biotechnology Center DNA Sequencing Facility using the BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) DNA sequencing system. Reaction products were analyzed on an Applied Biosystems 3730xl automated DNA sequencer. Sequencing reaction results for complementary strands were assembled and edited using Lasergene 5.0 (DNAStar, Madison, WI).

Comparative ITS and SSU sequences were selected through BLAST search hits to query WNS strain sequences from similar sequences archived in GenBank. Se-AL (v2.0a11) (Rambaut 2002) was used for visual alignment of the sequences. An ITS alignment of 537 nt for 20 taxa and a SSU alignment of 1725 nt for 18 taxa are archived in TreeBase SN3954-18967, with a substitution of identical sequences for EU884921 for EU854571; and FJ231093 for FJ231098, respectively. We determined parsimony phylograms using PAUP* (4.0b10) (Swofford 2002), and reliability of nodes with Bayesian posterior probabilities calculated with MCMC (MrBayes 3.1.2) (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) using the GTR model and running four chains with 1,000,000 generations, sampling each 1,000th tree and discarding as burn-in all pre-convergence trees. Bootstrap percentages were based on 1,000 replicates in PAUP* (Swofford 2002).

Results

DNA sequences and phylogenetic analyses

SSU (FJ231098) and ITS (EU884921) DNA sequences for the holotype are archived in GenBank; sequences archived for the paratypes are listed in Table 1; all SSU and all ITS sequences were identical. Although excluded from the sequences used in analysis, the SSU sequences also contain a putative optional group I intron of ca 415 nt, located at small subunit position 1506 (Gargas et al. 1995), with 97% sequence similarity to insertions in *Geomyces* spp. AY345348 and AY345347. Phylogenetic analyses of nuclear rRNA gene SSU (Fig. 1) and ITS (Fig. 2) sequences produced 5 equally parsimonious trees for the SSU and 13 equally parsimonious trees for the ITS, both supporting a close relationship between this fungus and species within the genera *Geomyces* and *Pseudogymnoascus* (Blehert & al. 4/27/2009: http://www.sciencemag.org/cgi/content/full/sci;1163874/DC1).

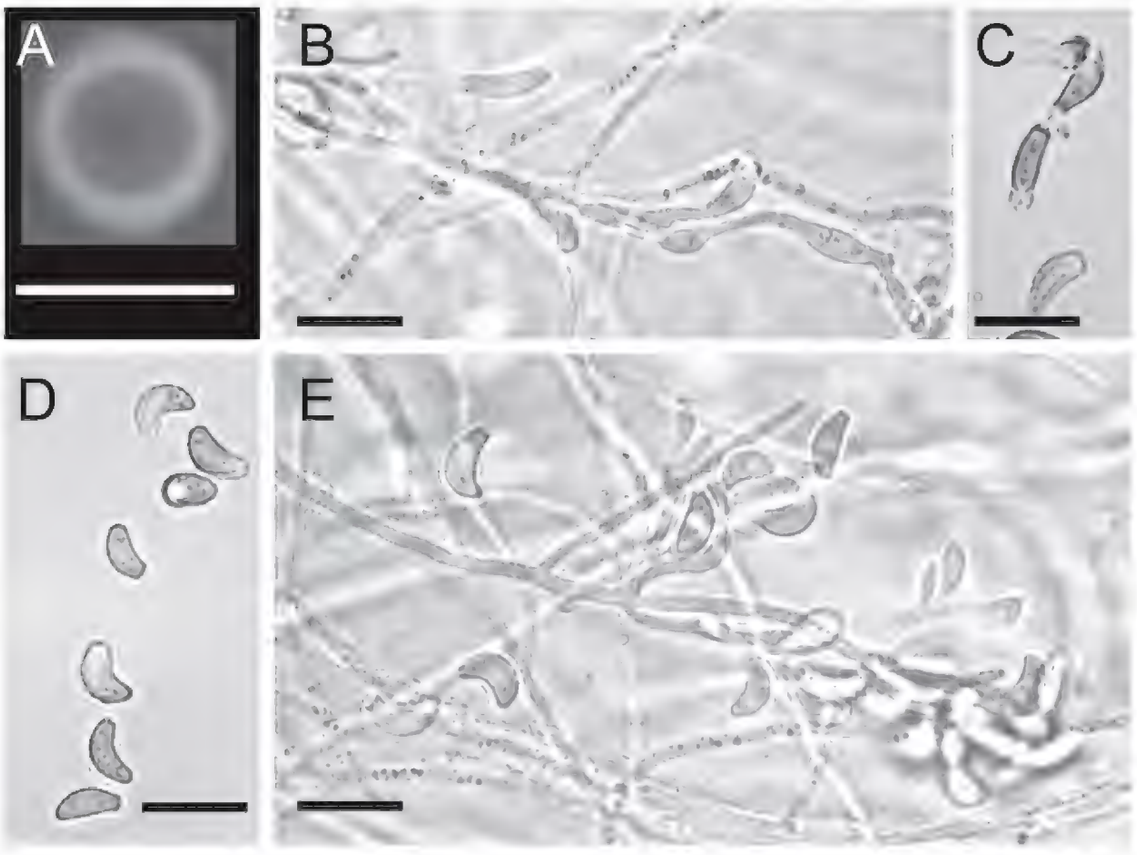

Fig. 3. *Geomyces destructans.* A. Colony on CMA after 16 days at 7°C. B. Conidiophores with conidia in short chains. C. Conidia with separating cell. D. Curved conidia. E. Conidiophores showing acute branching. Scale bars: A = 1 cm, B–E = 10 µm.

Taxonomy

Geomyces destructans Blehert & Gargas, **sp. nov.** Figure 3

MycoBank 513275; GenBank FJ231098, EU884921

Coloniae lente crescendens et psychrophilicae; nullum augmentum ad 24°C. Conidia acervulata pulverulenta, olivaceogrisea. Coloniae incoloratae vel sordidae brunneae ad reversum. Conidiosporae solitariae vel breves catenulatae, plurimum apicales, in erectis angustis verticillatis ramosis conidiophoris. Conidia thallica (arthroconidia) rhexolytica cum conspicuis cellis secedens, glabra aut prope glabra, plurima fortiter incurvata 5–12 × 2.0–3.5 µm, attenuata ad fundamenta et apicaliter ad 0.5–2.0 µm, truncata ad extremo una aut uterque, parce crassitunicata. Pathogenica ad chiropteras.

Type — USA. New York, Williams Hotel Mine, isolated from a wing of a little brown bat (*Myotis lucifugus*), 2 Feb 2008, Al Hicks [NHWC 20631-21] (**Holotype** BPI87895; ex-type culture NHWC 20631-21).

Etymology: *destructans* = destroying

Colonies on CMA (Fig. 3A) and Sab dex agar are slow growing and psychrophilic; colony diameter after 16 days 1.0 mm at 3°C, 5 mm at 7°C, 8 mm at 14°C, no growth at 24°C. Colonies are white marginally and with sterile white overgrowth centrally on Sab dex agar; conidial masses at colony centers

powdery, Gray to Gray-Green near Grayish Olive to Andover Green (Ridgway 1912, XLVI and XLVII); colony reverse uncolored on CMA, becoming Drab to Hair Brown (Ridgway 1912, XLVI) on Sab dex agar.

On CMA, asymmetrically curved conidia borne singly at the tips, on the sides, or in short chains on verticillately branched conidiophores (Fig. 3B). Intercalary conidia (arthroconidia) sometimes with conspicuous separating cells within chains of conidia (Fig. 3C), that undergo rhexolytic dehiscence. Conidiophores are erect, hyaline, smooth and thin-walled, narrow, 1.5–2 µm wide by 35–90 µm or more in length, commonly bearing verticils of 2–4 branches borne at an acute angle to the stipe (Fig. 3E). Branches may appear slightly sinuous due to the presence of curved conidia. Conidia are 5–12 × 2.0–3.5 µm, tapering basally to 1.5–2.0 µm and apically to 0.5–1.5 µm, truncate with prominent scars at one or both ends, smooth and lightly pigmented; predominantly curved, sometimes oval, obovoid, or cymbiform, moderately thick-walled at maturity and readily seceding (Fig. 3D).

Pathogenic to bats.

Additional strains examined: Seven paratypes are listed in Table 1.

Discussion

The outstanding characteristics of *Geomyces destructans* are conidium shape, very slow growth on artificial media, and cold-adaptation with no growth at 24°C or above. This fungus has currently only been identified from tissues of bats, where it invades living tissue (Meteyer et al. 2009) with associated high mortality. Through a combination of traditional morphological studies and molecular analyses we have identified the causal agent of white-nose syndrome cutaneous infection as a new species of *Geomyces: G. destructans.*

Species of *Geomyces* are known from soil worldwide, often from colder regions (see Carmichael 1962, Sigler & Carmichael 1976, Van Oorschot 1980, Sigler et al. 2000, Rice & Currah 2006, Kochkina et al. 2007). In our literature search for species with curved, fusiform conidia in *Geomyces* and closely related genera and among anamorphs of *Pseudogymnoascus*, we found one report of a strain with conidia often or sometimes curved. Among 11 strains of *Geomyces pannorum* from arctic cryopegs and surrounding marine deposits in northern Siberia, Kochkina et al. (2007) isolated a strain (FW-2264) with conidia "often curved, 4.4 × 3.1 µm, scar 1.5 µm". Additionally, this strain was slow growing and was psychrophilic, with spore germination 60% at –2°C and optimum growth at approximately 4°C. In contrast to *Geomyces destructans,* growth at 26°C was about 85 % of that at 4°C and presumably the conidia were echinulate and mostly obovoid.

White-nose syndrome has caused a devastating epizootic among bats of the northeastern US (Blehert et al. 2009), and the disease continues to spread

rapidly. This fungus grows optimally at the temperatures found in winter bat hibernacula. Bats are thought to have lowered immune responses during hibernation torpor (Carey et al. 2003), which may predispose them to infection by *G. destructans*.

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